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Aspergillus flavus — primary causative agent of aflatoxins in dried figs

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Abstract — *Aspergillus* sect. *Flavi* isolates from Turkish dried figs have been determined using PCR amplification of the ITS1 and ITS2 rDNA regions. Species were identified by comparing partial 18S rDNA sequences from 57 different fungal isolates comprising 5 *A. parasiticus* strains, 1 *A. tamarii* strain, and 51 *A. flavus* strains with known ribosomal sequences using BLAST search. Sequence comparisons between the isolates and reference cultures showed a 95 99% similarity; morphological and phenotypical character comparisons of the same strains produced equally close similarities. The sole exception was an aflatoxin B_1 and B_2 producing strain that cannot produce cyclopiazonic acid; this strain, originally identified as *A. flavus*, was instead found to represent *A. parasiticus*.

Key words *Aspergillus parasiticus*, Polymerase Chain Reaction, dried fruit, ITS, CPA

Introduction

The *Aspergillus flavus* group commonly found in nature is one of the most widely studied fungal species. *Aspergillus flavus, A. parasiticus, A. nomius, A. pseudotamarii,* and *A. bombycis* produce aflatoxin, a potent carcinogenic toxin, while *A. oryzae, A. sojae,* and *A. tamarii* are non-toxigenic species widely used in the production of fermentation products throughout Asia (Frisvad et al. 2007). Although these fungi are commonly used to produce enzymes and fermentation products, some cause serious problems through contamination of food and foodstuffs.

Aflatoxins (AFs), naturally occurring secondary metabolites, are potent hepatotoxic, mutagenic, and carcinogenic toxins causing serious health hazards

in humans and animals. Aflatoxin B_1, B_2, G_1 and G_2 are found predominantly in food whereas M_1 and M_2 aflatoxins are found primarily in animal tissues and fluids (milk, urine) as the hydroxylated metabolic products of aflatoxins B_1 (AFB_1) and B_2 (AFB_2) respectively (Richard 2007). Special interest is given to AFs due to their high occurrence and toxicity. The most toxic aflatoxin known, AFB_1, is cited as a group I carcinogen by the International Agency for Research on Cancer (IARC 1993). The maximum levels of AFB_1 and total aflatoxins allowable in dried fruits as determined by Commission of the European Communities are 2 and 4μg/kg respectively. Aflatoxigenic fungi occur in such food commodities as corn, peanuts, cottonseed, nuts, spices, figs, and dried fruits (Pitt & Hocking 1997). Aflatoxigenic fungi may contaminate foods throughout several stages (pre-harvest, processing, transportation, storage) of the food chain (Manonmani et al. 2005). The level of mould infestation and identification of species are important indicators of raw material quality and predictors of the potential risk of mycotoxin occurrence (Shapira et al. 1996).

Fungi in foods can be identified using traditional morphological and phenotypic observations of cultures in different media. However, such methods are time consuming and laborious and require mycological expertise and special facilities. Also, because morphological characters can vary over time, they should be verified by the mycotoxigenic profile of the strains (Samson et al. 2007). Furthermore, traditional methods do not distinguish toxigenic from non-toxigenic forms. For these reasons, application of molecular techniques has become increasingly important in detecting and characterizing potential contaminants within the food-processing industry. These methods are very rapid, specific, and sensitive compared to traditional methods.

There are two approaches in identifying aflatoxigenic fungi through PCR-based methodology. The first uses standard PCR to detect aflatoxigenic fungi in foods. In this technique, the contiguous Internal Transcribed Spacer (ITS) regions in fungal DNA fungi are amplified, sequenced, and compared extensively with sequences in GenBank (Hinrikson et al. 2005; Rodrigues et al. 2007; González-Salgado et al. 2008). The second approach is especially useful for the strains having nearly identical genes, as the PCR reaction targets six aflatoxin biosynthesis genes, namely; aflatoxin regulatory genes (AFLR, AFLS), norsolorinic acid reductase (NOR-1 = AFLD), versicolorin A dehydrogenase (VER-1) sterigmatocystin-o-methyltransferase (OMT-A), polyketide synthase (PKSA), and APA-2 in *A. flavus* and *A. parasiticus* (Shapira et al. 1996; Somashekar et al. 2004; Manonmani et al. 2005; Rodrigues et al. 2009). We used the first approach to detect ITS sequences from 57 *Aspergillus* sect. *Flavi* isolates, which had been previously characterized according to their morphological and phenotypic characters, from Turkish dried figs. This is the first report of molecular analyses of *Aspergillus* sect. *Flavi* strains isolated from a Turkish agricultural commodity.

Materials & methods

Fungal isolates and culture conditions

Fifty-seven strains of *A. flavus*, *A. parasiticus,* and *A. tamarii,* isolated from dried figs from the west of Turkey (Aegean region), were selected. The isolates had been previously morphologically and phenotypically identified to species level (Heperkan & Karbancıoglu-Güler 2009). All fungal cultures were maintained on slant malt extract agar (MEA) and stored at 4°C. Incubation was performed at 25°C for 5 days. Cultures were inoculated with 1 cm diameter mycelial disks cut from the plates and incubated in 30 ml Erlenmeyer flasks containing 20 ml of Potato Dextrose Broth (PDB) at 25°C and 150 rpm. Mycelia from 2-day-old cultures were harvested by filtration through Whatman No.1 paper and kept at –80°C for DNA isolation.

Fungal DNA extraction

Template DNA was extracted from 50–100 mg (wet weight) of fungal mycelia harvested from freshly growing cultures in PDB under stationary conditions. DNA was isolated by using ZR fungal / bacterial DNA system (purchased from Zymo Research, Orange, CA, USA) according to the manufacturer's protocol.

PCR amplification

DNA was amplified by PCR using fungal-specific 18S rDNA primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). The PCR amplification protocol was as follows: 1 cycle of 95°C for 5 min, 30 cycles of a set including 95°C for 1 min, 55°C for 1 min and 72°C for 1 min 40 s and a final cycle of 72°C for 10 min. Amplification of fungal DNA was carried out in a total reaction volume of 50 µl, containing 1 µl of template DNA, 1 µl of each primer, 5 µl of 10 x PCR buffer, 4 µl of $MgCl_2$ (25 mM), 1 µl of dNTPs and 0.5 µl of Taq DNA polymerase. The PCR reaction was performed using Biometra T Professional Thermocycler (Biometra, Goettingen, Germany).

DNA sequencing & analysis

Sequencing was carried out using an ABI PRISM 3100-Avant automated sequencer at the Molecular Biology and Genetics Department, ITU. Both strands of the PCR product were sequenced with the same primers. The resultant nucleotide sequences were aligned with MEGA sequence analysis software version 4.0 (Tamura et al. 2007).

Species were identified by comparing the partial 18S rDNA sequences with known ribosomal sequences using BLAST (Basic Local Alignment Search Tool). Comparisons of reference strain and isolate sequences were performed using Clustal W alignment algorithm. Sequence variation and similarity between sampled sequences were determined with MEGA 4.0 software; sequences were visually confirmed using pairwise nucleotide alignments.

Results & discussion

ITS regions from 57 *Aspergillus* sect. *Flavi* strains were sequenced to identify the species at the molecular level. ITS regions, located between the 18S and 28S RNA genes, offer distinct advantages over other molecular regions due to the existence of approximately 100 copies per genome. Amplification of the ITS

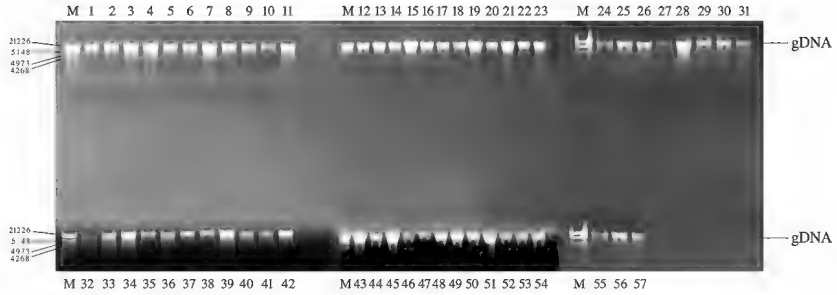

FIGURE 1. Agarose gel analysis of PCR products from genomic DNA of fungal cultures. Lane M, DNA molecular size marker (Lambda/HindIII); Lanes 1–7, 9–24, 26–28, 30, 32–35, 37–41, 43–57, *A. flavus*; Lanes 8; 25; 31; 36; 42, *A. parasiticus*; Lane 29, *A. tamarii*.

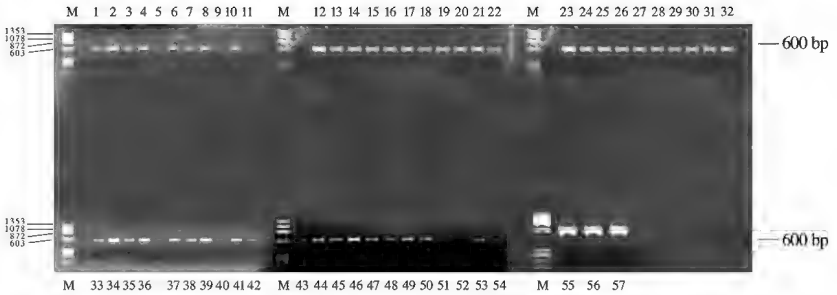

FIGURE 2. Agarose gel analysis of PCR amplification products from strains using ITS1-ITS4 primers. Lane M, PhiX174 DNA/BsuRI HAEIII marker; Lanes 1–7, 9–24, 26–28, 30, 32–35, 37–41, 43–57, *A. flavus*; Lanes 8, 25, 31, 36, 42, *A. parasiticus*; Lane 29, *A. tamarii*.

regions from the samples generated PCR products around 600 bp long. FIGURES 1 and 2 show the results of gel electrophoresis for genomic DNA isolation and PCR amplification, respectively.

A matrix comparing the ITS1 and ITS4 sequence similarities among the four *Aspergillus* reference species is depicted in TABLE 1.

Since the strains were previously identified as *Aspergillus* sect. *Flavi* using morphological and phenotypical criteria (Heperkan & Karbancioglu-Güler 2009), only *Aspergillus* sect. *Flavi* species were used for reference. Similarities between the isolate ITS sequences and the reference strain sequence of the same *Aspergillus* species are shown in TABLE 2.

We followed protocols set forth by González-Salgado et al. (2008) for differentiating *A. flavus* and *A. parasiticus* species in wheat flour. Although several PCR-based methods use aflatoxin biosynthetic genes to detect

TABLE 1. Matrix of ITS region similarities (%) for *Aspergillus* reference species

Aspergillus spp. (isolate codes) / base pairs (bp)	*A. flavus*	*A. oryzae*	*A. parasiticus*	*A. sojae*	*A. tamarii*	*A. pseudotamarii*
A. flavus (ATCC 16883) / 595						
A. oryzae (ATCC 4814) / 555	95					
A. parasiticus (NRRL 2999) / 576	98	96				
A. sojae (IFO 4386) / 576	98	96	99			
A. tamarii (JCM 2259) / 579	97	95	98	98		
A. pseudotamarii (NRRL 25517) / 598	97	91	95	95	95	

TABLE 2. Molecular identification of *Aspergillus* sect. *Flavi* isolates

No.	ITS1 / ITS4 sequence similarities (out of 5)	Similarity (%)	Isolate name	No.	ITS1 / ITS4 sequence similarities (out of 5)	Similarity (%)	Isolate name
1	3 / 3	99	*A. flavus*	30	2 / 2	98	*A. flavus*
2	3 / 3	99	*A. flavus*	31	2 / 1	96	*A. parasiticus*
3	3 / 3	99	*A. flavus*	32	2 / 1	95	*A. flavus*
4	3 / 3	99	*A. flavus*	33	3 / 2	99	*A. flavus*
5	3 / 3	99	*A. flavus*	34	2 / 2	97	*A. flavus*
6	3 / 3	99	*A. flavus*	35	1 / 4	95	*A. flavus*
7	3 / 3	99	*A. flavus*	36	3 / 3	99	*A. parasiticus*
8	3 / 3	98	*A. parasiticus*	37	2 / 3	95	*A. flavus*
9	3 / 3	99	*A. flavus*	38	4 / 2	99	*A. flavus*
10	3 / 3	99	*A. flavus*	39	3 / 5	99	*A. flavus*
11	3 / 3	99	*A. flavus*	40	2 / 2	97	*A. flavus*
12	3 / 3	99	*A. flavus*	41	2 / 3	97	*A. flavus*
13	3 / 3	98	*A. flavus*	42	1 / 3	98	*A. parasiticus*
14	3 / 3	98	*A. flavus*	43	1 / 2	99	*A. flavus*
15	2 / 2	95	*A. flavus*	44	2 / 2	97	*A. flavus*
16	2 / 2	98	*A. flavus*	45	2 / 3	99	*A. flavus*
17	2 / 2	95	*A. flavus*	46	2 / 3	98	*A. flavus*
18	2 / 1	96	*A. flavus*	47	3 / 2	99	*A. flavus*
19	2 / 2	95	*A. flavus*	48	3 / 2	99	*A. flavus*
20	3 / 1	98	*A. flavus*	49	2 / 2	95	*A. flavus*
21	2 / 3	98	*A. flavus*	50	3 / 3	99	*A. flavus*
22	3 / 2	99	*A. flavus*	51	3 / 3	98	*A. flavus*
23	2 / 2	97	*A. flavus*	52	2 / 2	99	*A. flavus*
24	3 / 5	99	*A. flavus*	53	3 / 2	99	*A. flavus*
25	3 / 1	95	*A. parasiticus*	54	2 / 1	97	*A. flavus*
26	3 / 3	99	*A. flavus*	55	3 / 4	99	*A. flavus*
27	3 / 2	99	*A. flavus*	56	2 / 1	98	*A. flavus*
28	1 / 3	95	*A. flavus*	57	2 / 2	98	*A. flavus*
29	3 / 3	99	*A. tamarii*				

aflatoxigenic fungi, it has been always a major problem to separate *A. flavus* from *A. parasiticus,* as the latter is always more easily amplified. It is believed that this might explain the high similarity in the specific primers used to amplify aflatoxin biosynthesis genes. However, ITS amplification allows us to differentiate these closely related species with greater sensitivity than single copy genes. The ITS region, which has been used for phylogenetic analysis for almost 20 years, offers distinct advantages because it customarily displays sequence variation between species as well as the minor variations within the strains of the same species. One disadvantage, however, is that the ITS region cannot differentiate toxigenic from non-toxigenic species. Potential toxigenicity of the fungi is better assessed by either monitoring several genes involved in the toxin biosynthetic pathway or analyzing mycotoxins using HPLC (Urano et al. 1992, Stroka & Anklam 2000), LC-MS (Rundberget & Wilkins 2002), and/or LC-MS/MS (Spanjer et al. 2008).

González-Salgado et al. (2008) were the first to attempt to detect *A. flavus* and *A. parasiticus* using a multicopy region (ITS). We used specific primers to amplify ITS1 and ITS2 conserved 18S rDNA regions and compared the results with the reference cultures. González-Salgado et al. (2008) initially had difficulties in differentiating *A. flavus* from *A. oryzae* and *A. parasiticus* from *A. sojae* due to the small (1–2 bp) difference separating these species. To eliminate this problem, we duplicated the amplification of some isolates. The molecular identifications were tested with 57 different fungal-isolates shown in Table 2. Results compared with the reference cultures showed a 95–99% similarity.

Henry et al. (2000), Geiser et al. (2007), Sanchez-Hervas et al. (2008), and Karthikeyan et al. (2009) have all noted the ability of molecular techniques to differentiate *Aspergillus* species. For example, *Aspergillus* sect. *Flavi* and *Nigri* species isolated from cocoa beans were first identified macro- and microscopically and the identity of the different isolates was confirmed through 5.8S-ITS rDNA sequencing after PCR amplification using the universal primers ITS4 and ITS5. *Aspergillus* spp. (including *A. flavus* and *A. tamarii*) and *Penicillium* spp. thought to represent different species were isolated and identified by ITS sequencing (Sanchez-Hervas et al. 2008).

Genetic variability among the *A. flavus* isolates was investigated by PCR amplification (using ITS1 and ITS4 primers) and sequencing of the ITS1, ITS2, and 5.8S rRNA regions from *A. flavus* isolates, yielding products of approximately 580 base pairs in length. Nucleotide sequences were compared with *A. flavus* ITS sequences in the Genbank database. Analysis of the genetic coefficient matrix derived from the ITS nucleotide sequences from *A. flavus* isolates showed that % similarities among the tested *A. flavus* strains ranged from a minimum of 12% to a maximum of 99% (Karthikeyan et al. 2009).

This molecular approach is also used to identify *Aspergillus* species from human exudates in the health sector. For example, *A. flavus, A. fumigatus,* and *A. terreus* — the most frequent causes of invasive mould infections in immunocompromised patients — have been identified by amplification of ITS1 and ITS2 conserved regions of 18S and 28S rDNA (Henry et al. 2000). Clinical application of this approach allowed the earlier diagnosis and selection of effective antifungal agents for patients with invasive aspergillosis. Most recently, serial specimens from 26 patients with documented fungal infections have been investigated by using the PCR-based ITS2 region amplification. The fungal pathogens identified were different *Aspergillus* and *Candida* species, *Rhizopus oryzae* and *Fusarium oxysporum* (Landlinger et al. 2009).

Molecular techniques have also been widely applied in an attempt to differentiate aflatoxigenic and non-aflatoxigenic strains of *Aspergillus* species. Morphological, chemical, and molecular characterizations of 31 *Aspergillus* sect. *Flavi* isolates from Portuguese almonds were used to identify aflatoxigenic and non-aflatoxigenic strains. Morphological characterization classified 18 isolates as *A. parasiticus* and 13 isolates as *A. flavus*. Aflatoxins and cyclopiazonic acid (CPA) production of isolates investigated for the chemical characterization revealed that all *A. parasiticus* isolates were strong AFB and AFG producers but did not produce CPA. Furthermore, 8% of *A. flavus* isolates were found to produce all 3 mycotoxin groups (i.e., AFB_1, AFB_2, CPA), while 77% of *A. flavus* isolates were classified as atoxigenic. Two genes of the aflatoxin biosynthetic pathway [AFLD (NOR1), AFLQ (ORD1=ORDA)] were investigated using molecular techniques. Although the AFLD gene did not show any correlation with aflatoxigenicity, AFLQ did show some correlation. AFLD and AFLQ are not the only genes to distinguish the toxigenicity of *Aspergillus* sect. *Flavi* species. Three other genes — VER-1, OMT-1, and APA-2 coding for key enzymes and a regulatory factor in aflatoxin biosynthesis — have been used to detect *A. flavus* and *A. parasiticus* species in grains and foods. Good results have been obtained only with the DNA of the aflatoxigenic moulds, namely *A. parasiticus* and *A. flavus* (Shapira et al. 1996).

Different food and feed commodities from Mysore City in India have been examined to isolate aflatoxin-producing fungi and assess aflatoxins in commodities by targeting the AFLR and OMT aflatoxin biosynthesis genes. Although useful for detecting the *A. flavus* group, however, this protocol did not distinguish aflatoxin producers from non-producers (Somashekar et al. 2004). Another Indian study used PCR-based amplification of the aflatoxin regulatory gene AFLR for rapid detection of aflatoxigenic fungi in groundnuts and maize (Manonmani et al. 2005).

Our molecular analyses have produced results comparable to those obtained by the identification of the same strains using morphological and

phenotypical characters by Heperkan & Karbancıoglu-Güler (2009). We did detect a difference for one isolate — the aflatoxin B_1 and B_2 producer strain originally identified as *A. flavus* (isolate 36) was in fact shown by sequence analyses to represent *A. parasiticus* (Oktay & Heperkan unpublished data). The mycotoxigenic character of isolate 36 was re-analyzed with HPLC, which showed that it produced aflatoxin B_1 and B_2 but not CPA, so describing isolate 36 as an aflatoxin B_1 and B_2 producer would be more appropriate. Our result does not agree with related literature and serves as a first indication of an unusual mycotoxin pattern in *A. parasiticus*.

In conclusion, the contiguous ITS region is a very useful approach for verification of morphologically and phenotypically pre-identified *Aspergillus* sect. *Flavi* species.

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